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13. ABSTRACT (Maximum 200) <p>The aim of this proposal is to test vaccines expressing mouse mutant or wild-type p53 for induction of protective immunity against challenge with tumor cell lines expressing either mutant or high levels of wild-type p53. The long term goal is to develop an efficacious vaccine with broad applicability for the treatment of human breast cancer patients. Our studies to date show that vaccinia virus recombinants expressing full-length wild-type p53 provide up to 70% protection to one of the model tumor cell lines under investigation. Vaccine efficacy can be improved by using mouse IL-12 as an adjuvant. This combination treatment also results in complete regression in approximately 40% of mice carrying already established tumors. Tumor rejection (with or without IL-12) is mediated by CD4+ and CD8+ T cells as well as by NK cells. The vaccine was less efficacious against some other tumor cell lines that expressed mutant p53 or high levels of wild-type p53. DNA vaccines expressing varied forms of wild-type or mutant p53 were also shown to inhibit the growth of some but not all tumors overexpressing p53. Additional experiments to improve the efficacy of the vaccines to the more resistant tumors have been initiated.</p>				
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FOREWORD

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(5) INTRODUCTION

Vaccination is the most effective medical intervention to reduce human morbidity and mortality. While vaccines to microbial agents have been used successfully for decades to reduce the incidence of infectious diseases, efficacious vaccines to cancer have been scarce, partially due to a lack of suitable 'tumor specific' antigens. With advances in molecular oncology, protein alterations are being identified at a rapid rate in transformed cells. These modifications can be caused by point mutations, alternate splicing or overexpression of normal gene products.

One of the most frequent alterations of human tumors are mutations or marked overexpression of the p53 protein, either of which can be found in more than 50% of the most common types of human cancers. Wild-type p53 serves as a tumor suppressor protein which regulates cell growth and induces apoptotic cell death upon severe cellular DNA damage. Mutations of p53, which cluster in well-defined hot spots of the gene, lead to structural changes and functional inactivation of the p53 protein by altering its DNA binding domain. The p53 protein is only functionally active upon formation of tetramers, mutations of a single p53 allele can therefore have a dominant negative effect on the correct protein synthesized by the wild-type allele. Most mutations also prolong the half-life of the protein resulting in overexpression.

Point mutations of self proteins can induce an immune response provided the amino acid exchanges are flanked by anchoring residues that are able to bind to MHC determinants. Peptides delineated from a mutated region of p53 were shown to induce in experimental animals a T cell mediated immune response to tumors expressing the homologous mutation (1,2). The immune response to individual epitopes is genetically restricted (3), thus limiting the usefulness of single epitope vaccines for outbred populations.

Overexpression of a self protein can cause exposure of so-called cryptic epitopes (4,5). T cells are negatively selected in the thymus during development, causing apoptotic cell death of T cells which carry receptors with high affinity to self epitopes. Additional extrathymic pathways ensure tolerance to immunodominant epitopes of self proteins. Cryptic epitopes are epitopes that at physiological expression levels have too low an affinity for MHC determinants to reach the threshold needed for induction of T cell tolerance. Upon over-expression of the protein or upon alteration of its processing, a sufficient amount of these low affine epitopes can associate with MHC determinants and, provided that presentation is mediated by professional antigen presenting cells within the context of lymphatic tissue, result in the induction of a T cell-mediated immune response.

Wild-type p53, a protein that is present at low levels in all nucleated cells throughout the body, was shown by several groups including ours within the realm of this program to induce a T cell-mediated immune response (6-8) in experimental animals upon expression by a vaccine. These findings suggest that wild-type p53 might serve as a highly suitable target antigen for active immunotherapy against a wide variety of tumors.

Different types of subunit vaccines, i.e., vaccines expressing a single antigen or a fragment thereof, can be developed. Recombinant viral vaccines, such as those based on vaccinia viruses (9), generally induce potent T and B cell-mediated immune responses against the inserted antigen. DNA vaccines, one of the latest additions to the field of vaccinology, also result in stimulation of antibodies, T helper cells, and cytolytic T cells (10-13). DNA vaccines were shown to induce T cell responses in non-responder haplotypes (14), indicating that antigen as expressed by a DNA vaccine might cause exposure of hidden (cryptic) epitopes which might be an advantage for vaccines to tumor-associated antigens such as p53.

Within the realm of this program we have been testing DNA vaccines and vaccinia virus recombinants expressing wild-type or mutant p53 for induction of protective immunity to challenge

with tumor cells expressing either mutant p53 or high levels of wild-type p53. The effector mechanisms that contribute to protection are being investigated.

(6) BODY

Tumor cell systems: A number of tumor cell lines were obtained. Transcripts of *p53* were amplified by reverse transcription polymerase chain reaction (RT-PCR) and sequenced to characterize potential mutations (Table 1). In addition, for some of the cell lines, the levels of p53 expression were established by indirect immunofluorescence or by Western Blot analysis using a p53 specific monoclonal antibody (Ab-1, Oncogene, Cambridge, MA or mAb-18). Cell lines were titrated in syngeneic mice upon s.c. inoculation of graded numbers of cells to establish the minimal tumorigenic dose (TD₁₀₀), defined as the number of cells that cause visible tumors in 100 % of control mice within 2-4 weeks. Some of the cell lines was characterized for secretion of TGF- β (experiment conducted by Dr. U. Rodeck, The Wistar Institute). The results are summarized in Table 1.

Table 1

Tumor line	Tumor type	Origin	p53 Type/Level	1 TD ₁₀₀	TGF- β secretion act./total (pg/ml)
Gl261	Glioma	C57Bl/6	wt*/++	1 x 10 ⁵	70 / 936
B16.F10	Melanoma	C57Bl/6	wt/n.t.	5 x 10 ⁴	14 / 176
CT26	Colo-Rectal CA	Balb/c	wt/++	5 x 10 ⁴	1 / 2075
MethA-34	Fibrosarcoma	Balb/c	mu*/++++	1 x 10 ⁶	n.t.
66.1	Mammary CA	Balb/c	wt/+++	4 x 10 ⁴	27 / 759
410.4	Mammary CA	Balb/c	wt/++	3 x 10 ⁴	51 / 3352
t(10)1rasE7E6	Transformed fibroblasts	Balb/c	none	2 x 10 ⁴	n.t.

wt* - wild-type p53, mu* - mutant p53 double mutations causing amino acid exchanges in position 168 (Glu to Gly) and 234 (Met to Isoleucin), 1 TD₁₀₀ - minimal dose of tumor cells that causes visible tumors within 2-4 weeks after subcutaneous inoculation of cells. TGF- β - active and inactive form.

We obtained a fibroblast line, termed (10)1, immortalized due to the spontaneous loss of both p53 alleles (15). This fibroblast line was transformed by a triple promoter retroviral vector (16) expressing v-Ha-ras under the control of the long terminal repeat, E7 of human papilloma virus (HPV)-16 under the control of the HSV promoter, and E6 of HPV-16 under the control of the SV40 promoter. Transformed cells, termed t(10)1rasE7.E6, were selected in vivo by passage in mice. This tumor cell line which lacks p53 expression is used as a control.

Vaccines to p53: Vaccinia virus recombinants expressing the full-length wild-type p53 (termed Vp53-wt) or different mutations (Vp53-mu135 -mutation at position 135 from phenylalanine to alanine, Vp53mu168.234 - double mutation at position 168 glutamic acid to glycine, and at position 234 methionine to isoleucin) of p53 were constructed and tested upon infection of p53 negative cells for expression of p53 transcripts by RT-PCR using the appropriate p53 primers and for protein expression by Western Blot analysis. DNA vaccines based on different plasmid vectors using the SV40 or the CMV promoter, such as the high expressing vector

pVR1012.2 (kindly provided by Vical, Inc.) in which we replaced the kanamycin resistance gene with the ampicillin resistance gene (which contains additional immunostimulatory CpG sequences, 17) were generated expressing full-length wild-type, mutant (135, or 168.234) or truncated (deletion of the 1-70 transactivation domain) p53. A recombinant baculovirus expressing wild-type p53 upon infection of insect cells, as determined by Western Blot analysis, was produced. A monoclonal IgM antibody which recognizes a linear epitope of the C terminus of p53 as determined by Western Blot analysis was also generated by using synthetic phosphorylated peptides coupled to a T helper cell epitope. This antibody, generated in collaboration with Dr. L Otvos, termed mAb-18, recognizes a phosphorylated epitope of p53 (18).

Immune response to vaccinia virus recombinants expressing p53. Most of our studies to date have been based on the Vp53-wt vaccine expressing full-length wild-type mouse p53.

Induction of protective immunity to challenge: Mice immunized with a single dose of 2×10^7 plaque forming units (pfu) of Vp53-wt were partially protected (i.e., 30-80 % complete protection, and delayed onset of tumors in the rest of the mice) against challenge with a minimal tumorigenic dose of the GL261 glioma cell line which caused tumors in 90-100 % of the control mice immunized with 2×10^7 pfu of a vaccinia rabies virus glycoprotein (VRG) recombinant based on the same strain of vaccinia virus (i.e., Vaccinia virus strain Copenhagen, ref. 9). Vaccination with the Vp53-wt vaccine did not protect against challenge with a 10 times higher dose of GL261 tumor cells. Nevertheless, Vp53-wt vaccinated mice that remained tumor-free after the initial inoculation with 1 TD₁₀₀ were then completely resistant to a subsequent challenge with 10-50 times more tumor cells that rapidly formed tumors in all of the VRG-immune control mice (see appendix and Table 2) but not against challenge with an unrelated p53-expressing tumor cell line. These data show that the tumor challenge had a booster effect in vaccinated mice which had then augmented resistance to further encounter with the same tumor cells. The efficacy of the Vp53-wt vaccine against growth of the GL261 cell line was not augmented by vaccinating mice with 2 doses of 2×10^7 pfu each of the Vp53-wt vaccine (data not shown), which presumably reflects neutralization of the second vaccine dose by antibodies to surface proteins of vaccinia virus. A similar level of protection to GL261 challenge was achieved with a vaccinia virus recombinant expressing a mouse mutant p53 protein (Vp53-mu135, data not shown).

We next tested in a series of experiments if vaccination with the Vp53-wt vaccine induced protection against other tumors. No protection could be achieved against the p53-negative tumor cell line, i.e., t(10)1rasE7E6, tested in BALB/c mice. Some protection could be achieved against wild-type p53 positive adenocarcinomas such as CT-26 (colon cancer), 66.1 and 410-4 (mammary tumors also tested in BALB/c mice). Protection was not as impressive as that seen in C57Bl/6 mice challenged with GL261 cells; the vaccine did not induce complete resistance in more than 10-30 % of mice, but resulted in a statistically significant delay of onset of tumor lesions. Lack of protection was not strictly correlated with levels of p53 expression, for example the 66.1 tumor cell line expresses higher levels of p53 than the GL261 cell line. Characteristics of the individual tumor cell lines such as density of MHC class I expression, down-regulation of TAP-1/-2 or LMP2/7, expression of fas-ligand, and secretion of immunomodulatory molecules such as IL-10 or prostaglandin E (reviewed in 19) might affect the efficacy of the Vp53-wt vaccine. No correlation was seen between vaccine failures and levels of TGF- β secretion (Table 1). In addition, genetic difference between the two mouse strains, i.e., C57Bl/6 and BALB/c, are likely to influence vaccine induced resistance. For example, BALB/c mice, due to a postulated defect in the IL-12 pathway, are more prone to develop Th2 responses, which might be less effective in eliminating tumor cells. Furthermore, both mouse strains have a different MHC haplotype, which determines availability of suitable T cell epitopes (i.e., Ir-gene control).

Table 2

Vaccine	Cell Line	% Resistance	Onset of Tumors (days \pm SD)	Significance
VRG	GL261	9.7	28.5 \pm 15.1	0.000001
Vp53-wt	GL261	76.4	51.3 \pm 24.6	
VRG	B16.F10	0	23.5 \pm 4.1	0.035
Vp53-wt	B16.F10	0	30.4 \pm 7.8	
VRG	66.1	0	28.0 \pm 5.7	0.017
Vp53-wt	66.1	30	45.3 \pm 9.1	
VRG	410.4	0	18.9 \pm 3.2	0.008
Vp53-wt	410.4	12.5	24.6 \pm 1.5	
VRG	CT-26	0	39.8 \pm 1.7	0.003
Vp53-wt	CT-26	10	49.6 \pm 5.9	
VRG	t(10)1rasE7E6 0		19.2 \pm 2.9	0.038
Vp53-wt	t(10)1rasE7E6 0		16.4 \pm 1.7	

The table shows the summary of several experiments. Protection to GL261 cells was tested in 5 separate experiments in a total of 31 (VRG) or 34 (Vp53-wt) mice. Groups of mice (minimal number : 8) were immunized with 2×10^7 pfu of VRG or Vp53-wt virus. Two weeks later mice were challenged with 1-2 TD₁₀₀ of the different tumor cells. Mice that failed to develop tumors within the observation periods (50-100 days) were scored as resistant. For mice that developed tumors, the mean day from challenge until visible onset of tumor growth (>2 mm in diameter) \pm standard deviation was recorded. Significance of difference between onset in VRG and Vp53-wt vaccinated mice was calculated by student τ test.

Immune effector mechanisms involved in providing protection to tumor challenge: The immune effector mechanisms that provide protection upon Vp53-wt vaccination were determined in knock-out mice. The following four knock-out mouse strains were used: CD4-KO mice (20) to test for a requirement for T helper cells, β 2 microglobulin-KO mice (21) that lack MHC class I expression and hence CD8⁺ T cells to test for a putative role of cytolytic T cells, perforin knock-out mice (22) to determine a role for cytolysis via the perforin pathway, and μ MT mice (23), that due to a immunoglobulin heavy chain transmembrane defect lack functional B cells to establish a potential contribution of antibodies in mediating tumor rejection. Neither CD4KO mice nor β 2KO mice could be protected by the Vp53-wt vaccine against challenge with the GL261 cell line, indicating that both T cell subsets were needed to limit the growth of the GL251 cell line. In

contrast, μ Mt mice showed only a slight reduction in resistance and perforin KO mice showed the same level of protection upon vaccination as wild-type C57Bl/6 mice, suggesting that antibody-mediated immune effector mechanisms do not play a major role in providing protection and perforin-mediated cytotoxicity is not needed for the elimination of GL261 tumor cells.

To further ascertain that protection required both CD8⁺ and CD4⁺ T cells and to furthermore establish if CD4⁺ T cells had to be present during the induction phase (i.e., shortly after vaccination) to provide help to CD8⁺ T cells or if CD4⁺ T cells were also needed during the effector phase (i.e., at the time of tumor challenge), antibody depletion studies were conducted using the monoclonal antibodies GK1.5 (rat anti CD4) and 53-6-72 (rat anti-CD8). In the initial experiment, mice were treated with the antibodies prior to vaccination. In a subsequent experiment, mice were first vaccinated and then 2 weeks later treated with the antibodies at the time of tumor challenge. Regardless of the timing, depletion of either CD4⁺ or CD8⁺ T cells resulted in loss of protection. In addition, mice that were depleted of either subset showed accelerated growth of tumors which was particularly pronounced after depletion of CD4⁺ T cells. Natural killer cells were also required for limiting tumor growth in Vp53-wt vaccinated GL261-challenged mice as depletion of this subset by treatment with a rabbit serum to asialo-GM-1 completely abrogated protection.

Table 3

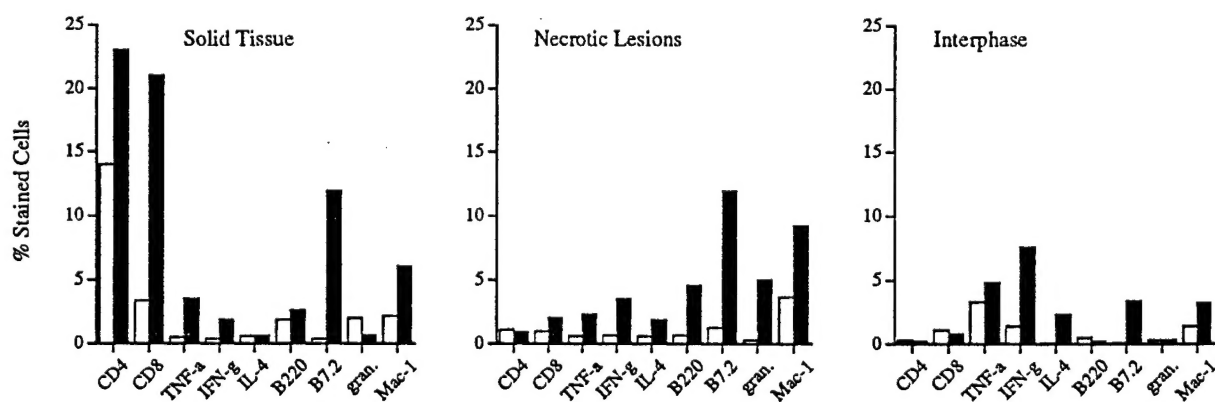
Vaccine	Recipient	Tumor Free Interval (days \pm SD)	% Resistance
VRG	C57Bl/6	28.5 \pm 15.1	10
Vp53-wt	C57Bl/6	51.3 \pm 24.6	75
Vp53-wt	CD4 depleted	14.9 \pm 2.6	0
Vp53-wt	CD4KO	22.0 \pm 5.5	33
Vp53-wt	CD8 depleted	23.1 \pm 2.2	0
Vp53-wt	B2m	30.1 \pm 8.1	3
Vp53-wt	NK depleted	20.4 \pm 5.4	0
Vp53-wt	μ MT	45.2 \pm 19.1	50
Vp53-wt	perforin-KO	40.0 \pm 0	75

Mice were vaccinated with VRG or Vp53-wt. They were challenged 2 weeks later with GL261 tumor cells. Lymphocyte subsets were depleted by injecting mice i.p. with the appropriate antibody on days -4, -1, +1, +4 and +8 with respect to tumor challenge.

Histochemical analysis of tumors: GL261 tumors from VRG or Vp53-wt vaccinated mice were analyzed for an inflammatory infiltrate by staining for cell surface markers on T helper cells (CD4), cytolytic T cells (CD8), B cells (B220), granulocytes, macrophages (mac-1), and activated antigen presenting cells (B7.2) using commercially available antisera or monoclonal antibodies. Frozen sections were stained using the avidin-peroxidase Vectastain Elite BC kit (Vector Laboratories). They were developed with 3',3'-diaminobenzidine tetrahydrochloride (Sigma) and counterstained with 1% HE. In addition, formalin fixed sections were stained for cytoplasmatic

expression of IFN- γ , IL-4 and TNF- α . Sections were analyzed in a SONY up-5500/5600 microscope with the computational ability to count the number of stained cells per section or to determine the % area of stained cells/total area of cells. The tumor sections derived from tumors of 1-2 cm in diameter showed three distinct areas: solid tissue which was fairly homogeneously infiltrated with mononuclear cells, necrotic areas with a more discrete infiltrate, and the interphase between solid and necrotic parts of the tissue which showed the most pronounced aggregation of mononuclear cells. Two to three representative sections were analyzed and the means of the obtained values are shown in Figure 1. Comparing tumors from VRG immune control mice and Vp53-wt vaccinated mice, the most striking observation was the increased influx of CD8 $^{+}$ T cells into the solid tissue. CD4 $^{+}$ T cells, already present at a high level in tumors of control mice, only increased about 2 fold in solid tissue. Only a few T cells were scattered throughout the necrotic area or at the interphase, where granulocytes, mac-1 $^{+}$ cells, and B220 $^{+}$ cells were more frequent. B7.2, a co-stimulatory molecule expressed on antigen-presenting cells such as dendritic cells, was mainly found in the solid tissue of Vp53-wt vaccinated mice concomitant with CD4 $^{+}$ and CD8 $^{+}$ T cells. Intracytoplasmatic staining for the cytokines, i.e., IFN- γ , IL-4, and TNF- α , showed an increase for all of them, less in solid tissue but mainly at the interphase and in areas of necrotic lesions. The most pronounced increase was seen for IFN- γ . Taken together these data indicate that CD4 $^{+}$ T cells play a major role in controlling tumor growth even without specific vaccination, massive excavation of CD8 $^{+}$ T cells is a consequence of Vp53-wt vaccination which also enhances (presumably as a bystander effect of antigen specific effector mechanism) recruitment of inflammatory cells such as granulocytes and macrophages. Specific vaccination also enhances influx of cytokine secreting cells, especially those secreting IFN- γ , a cytokine indicative for a Th1 type immune response (Figure 1).

Figure 1



Legend: Thin section of 1-2 cm in diameter GL261 tumors from Vp53-wt (■) or VRG (□) immunized mice were stained with antibodies to cell surface markers or cytokines in the cytoplasm. Several 3 sections of each tumor containing solid tissue, necrotic tissue, or the interphase between the two were analysed.

IL-12 has a synergistic effect to vaccination with Vp53-wt: In an effort to enhance vaccine efficacy, IL-12, a cytokine that promotes Th1 type responses and has known therapeutic effects against tumors which might in part be caused by its anti-angiogenic activity, was used as an adjuvant. Vp53-wt or VRG vaccinated mice challenged 2 weeks after immunization were treated at varied times after tumor inoculation with 0.25 µg/ mouse/ day of mouse recombinant IL-12 protein given i.p. for 4 consecutive days. IL-12 itself (i.e., in combination with the VRG control vaccine) resulted in partial protection, nevertheless, a combination of Vp53-wt and IL-12 had a synergistic effect causing complete protection in up to 90-100% of mice. The degree of protection depended on the time lapse between tumor challenge and IL-12 therapy. Best results were obtained when IL-12 was given shortly (within 1-6 days) after challenge. Depletion of CD4⁺, CD8⁺ or NK cells at the time of tumor challenge resulted in a loss of protection (Table 4).

Mice that were vaccinated with a combination of Vp53-wt and IL-12 or IL-12 only that survived the initial challenge with tumor cells were rechallenged with an increased dose of GL261 cells or with unrelated p53 expressing tumor cells, i.e., B16.F10 cells. Again, mice showed complete protection to challenge with GL261 cells but only partial protection (i.e., delay of onset of lesions) to B16.F10 cells. Mice vaccinated initially with the Vp53-wt vaccine and IL-12 showed the most pronounced protection to B16.F10 cell challenge with regard to mean delay of tumor onset (Table 5).

Table 4

Vaccine	Depletion of lymphocytes	IL-12, days of treatment	experiments 1+3		experiments 2 + 4	
			Tumor free interval (days)	% Protection	Tumor free interval (days)	% Protection
Vp53-wt	-	-	45 ± 16	63	42 ± 15	42
VRG	-	-	45 ± 24	14	23 ± 4	0
Vp53-wt	-	2-5	68 ± 17	75	34 ± 0	90
VRG	-	2-5	85 ± 7	75	41 ± 13	20
Vp53-wt	-	5-9	>100	100	44 ± 11	30
VRG	-	5-9	42 ± 4	50	41 ± 13	40
Vp53-wt	-	15-19	40 ± 0	63	64 ± 17	10
VRG	-	15-19	50 ± 14	42	35 ± 14	20
Vp53-wt	-	-	40 ± 8	30	54 ± 19	30
VRG	-	-	26 ± 8	0	29 ± 4	0
Vp53-wt	-	2-5	46 ± 0	90	>100	100
VRG	-	2-5	38 ± 16	70	48 ± 0	77
Vp53-wt	CD4	2-5	23 ± 10	10	21 ± 2	0
VRG	CD4	2-5	25 ± 9	0	17 ± 2	0
Vp53-wt	CD8	2-5	27 ± 10	10	43 ± 11	20
VRG	CD8	2-5	22 ± 8	20	26 ± 7	13
Vp53-wt	CD4/8	2-5	n.t.	-	18 ± 8	0
VRG	CD4/8	2-5	n.t.	-	16 ± 2	0
Vp53-wt	NK	2-5	n.t.	-	26 ± 12	0
VRG	NK	2-5	n.t.	-	38 ± 2	20

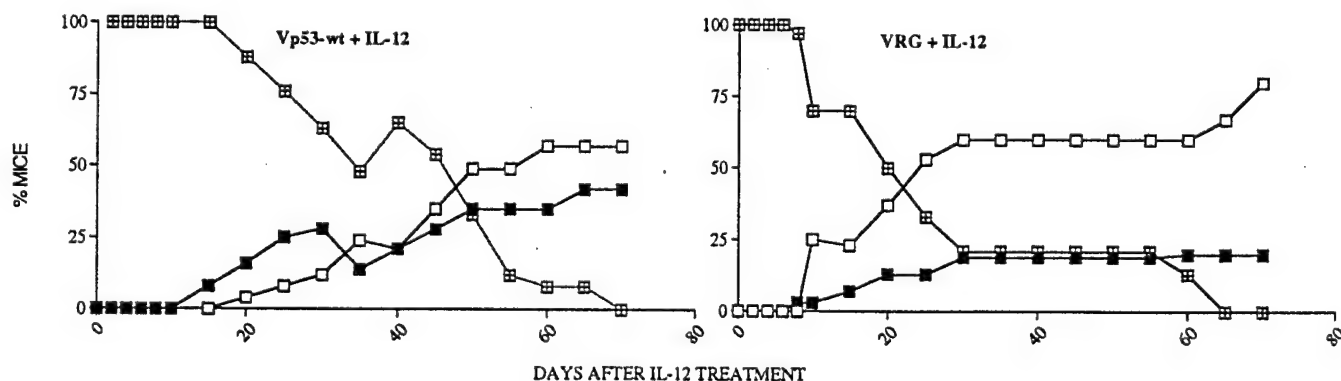
Groups of 8-10 mice were vaccinated with 2×10^7 pfu of Vp53-wt or VRG virus. They were challenged 14 days later with 1 TD₁₀₀ of GL261 cells. Onset of tumor lesions (mean days ± SD) and % complete protection from tumor development for an observation period of 100 days was recorded. In experiments 1 and 2 (upper part) the effect of IL-12 given at different times after challenge was tested. IL-12 was given at 0.25 µg per day i.p. for 4 days either on days 2-5, 5-9 or 12-15 following challenge. In experiments 3 and 4 (lower part) the effect of antibody-mediated depletion of lymphocyte subsets on vaccination followed by IL-12 treatment given days 2-5 following challenge was tested. Mice were depleted of CD4⁺ or CD8⁺ T cells or both or of NK cells by injecting the appropriate antibodies on days -4, -1, +1, +4 and +8 with regard to tumor challenge. n.t. - not tested

Table 5

Vaccine	IL-12	1st challenge	2nd challenge	mean tumor free interval % complete protection (n)*	
Vp53-wt	+	GL261	GL261	>50	100% (9)
VRG	+	GL261	GL261	>50	100% (8)
None	-	-	GL261	18.3 ± 3.2 /	0% (8)
Vp53-wt	-	GL261	B16.F10	23.3 ± 2.4 /	22% (9)
Vp53-wt	+	GL261	B16.F10	28.3 ± 4.0 /	11% (9)
VRG	-	GL261	B16.F10	22.7 ± 2.3	0% (3)
None	-	-	B16.F10	13.7 ± 2.6	0% (9)

Groups of mice were vaccinated with 2×10^7 pfu of the Vp53-wt or VRG vaccine. They were challenged 14 days later with 1 TD₁₀₀ of GL261 cells. Some of the groups were treated with IL-12 at 0.25 µg/day i.p. on days 2-5 following challenge. Mice that remained tumor-free were rechallenged with 10 TD₁₀₀ of GL261 cells or B16.F10 cells. Additional naive mice were also challenged at this time. Data show the mean tumor-free interval after the 2nd challenge in days ± SD and the % of mice that did not develop tumors over the 100 day observation period after the 2nd challenge. n - number of mice used for the 2nd challenge.

Figure 2



Legend: Mice were injected with GL261 cells s.c. Mice with clearly visible tumors (0.3 - 0.5 mm in diameter) were vaccinated with 2×10^7 pfu of the Vp53-wt or the VRG vaccine. The following day IL-12 treatment was initiated. The graphs show the summary of 3 experiments. In the first 2 experiments mice developed tumors rapidly and the experiments were terminated on day 35. In the 3rd experiment tumors developed more slowly and mice were observed for 70 days. Data are expressed as % mice of all 3 experiments that had small to medium size tumors (□), large tumors requiring euthanasia (□), or had no longer any evidence of tumors (■).

A combination of IL-12 and Vp53-wt causes regression of already established tumors: Cancer patients are not treated prophylactically but rather after the cancer is sizable enough for diagnosis. Immunotherapy is generally initiated after debulking of the tumor by surgery where possible, followed by conventional therapy. To test if the Vp53-wt vaccine in combination with IL-12 induced an immune response that could eliminate small existing tumors, groups of C57Bl/6 mice were first inoculated with 1 TD₁₀₀ of GL261 cells. Once the tumors became visible (0.3-0.5 mm in diameter) the mice were vaccinated either with the Vp53-wt vaccine or with the VRG construct. The following day IL-12 treatment was initiated for 5 days at 0.25 µg/mouse. As shown in Figure 2, the combination treatment of IL-12 and Vp53-wt vaccine resulted in complete regression of subcutaneous GL261 tumors in over 40% of mice; 2 of these 20 mice had a tumor recurrence later on, the others remained disease-free. IL-12 in combination with the control vaccine had a temporarily inhibitory effect on tumor growth and caused complete regression in only 20% of mice.

DNA tumor vaccines to p53. We generated a number of DNA vaccines expressing either full-length or truncated (removal of base pairs 1-70, i.e., transactivation domain) wild-type or mutant p53 (using the same mutations that were used for construction of vaccinia virus recombinants, i.e., single point mutation in position 135 and double point mutation in positions 168 and 234) and tested them for induction of protection against a subclone of a MethA tumor cell line expressing the same double mutation of p53 (135/268) as one of the DNA vaccines. Results were variable. In the experiment shown in Table 6, up to 70% complete protection could be achieved against the MethA-34 tumor cell line regardless of the form of p53 (wild-type or mutant) or the promoter driving expression of p53 (SV40 or CMV). In these experiments, mice that were completely protected remained resistant to further challenge with a 5 fold higher dose of the same tumor cell line while they remained susceptible to challenge with a high dose of an unrelated tumor cell line (i.e., CT-26) or a p53⁻ tumor cell line (i.e., t(10)lrasE7E6) (Table 7).

Table 6

Vaccine	Complete Protection
pVR1012.2	2 / 10
pVR1012.2p53mu168/234	7 / 10
pVR1012.2Dp53mu168/234	6 / 10
pSV2p53mu168/234	6 / 10
pSV2p53wt	6 / 10

Mice were immunized i.m. with 100 µg of DNA. They were challenged 3 months later with MethA-13 cells and tumor development was recorded.

Table 7

Mice	Challenge	Complete Protection
DNA vaccine + MethA-34	MethA-34	8 / 8
Naive	MethA-34	0 / 5
DNA vaccine + MethA-34	CT-26	0 / 9
Naive	CT-26	0 / 5
DNA vaccine + MethA-34	t(10)1rasE7E6	0 / 8
Naive	t(10)1rasE7E6	0 / 5

This experiment is a continuation of the one shown in Table 6. Mice that were immunized with the p53 expressing DNA vaccines and remained tumor-free after the 1st challenge were rechallenged with 10 TD₁₀₀ of different tumor cells. Tumor development was recorded.

In an additional experiment the DNA vaccine based on the pVR1012 vector expressing the 168/234 double mutation of p53 showed partial protection in C57Bl/6 mice against challenge with GL261 cells (Table 8). Significant protection could not be induced against other tumor cell lines such as the adenocarcinoma line 66.1 or the CT-26 colorectal carcinoma line, indicating that further modifications are needed to improve the efficacy of the DNA vaccine.

Table 8

Vaccine	% Complete protection	Tumor free interval (mean days \pm SD)
pVR1012.2	10	27.4 \pm 10.8
pVR1012p53mu168.234	30	32.9 \pm 10.4
pSV2p53mu135	11	27.2 \pm 13.0
pSV2p53mu168.234	20	27.4 \pm 8.5

Legend: Groups of 9-20 mice were injected with 50 μ g of the DNA vaccine. They were challenged with 1 TD₁₀₀ of GL261 cells. Data represent the percent of mice that remained completely tumor-free during the 50 day observation period as well as the mean tumor free interval after challenge (days \pm SD).

(7) CONCLUSIONS

Our data shows that vaccines expressing wild-type p53 can induce protective immunity reflected by complete resistance or delayed onset of tumor lesions to challenge with tumor cells expressing high levels of either wild-type or mutant p53. The efficacy could be increased by using IL-12 in combination with the vaccine. This regimen could induce complete regression of pre-existing tumors in ~ 45% of mice. Mice that were protected against the initial low-dose challenge remained completely resistant to an additional challenge with a higher dose of the same tumor cells. A number of immune effector mechanisms contribute to protection, most notably CD4⁺ and CD8⁺ T cells as well as NK cells. Different tumor cells showed distinct susceptibility to the p53-induced immune effector mechanisms; while the GL261 cell line was highly susceptible, others such as the B16.F10 melanoma cell line were resistant. We have not yet established which host or tumor cell parameters determine the efficacy of the p53 vaccine. We did not observe a correlation between susceptibility and levels of p53 expression (the susceptible GL261 cell line expresses less p53 than the more resistant 66.1 mammary adenocarcinoma cell line) or levels of TGF- β secretion or the H-2 haplotype (both the susceptible GL261 and the resistant B16.F10 cell lines are of C57Bl/6 origin). Other parameters such as: levels of MHC class I expression, secretion of IL-10, and susceptibility to T cell-mediated cytolysis are being investigated. We are also testing if we can improve the efficacy of the Vp53-wt vaccine to the more resistant tumors by using a combination treatment with recombinant mouse IL-12.

The efficacy of the p53 expressing DNA vaccines was overall disappointing. We hypothesize that p53 as expressed by a plasmid vector is mainly targeted to the nucleus and thus not available for presentation with MHC determinants. We are currently reconstructing the DNA vaccine by cloning a fusion gene composed of the signal sequence of adenovirus and a mutant p53 (mutated in position 338 to generate a biologically inactive form unable to form heterodimers). We expect that this fusion protein will be targeted towards the endosomal pathway, thus available for presentation with MHC class I determinants, which will result in an improved CD8⁺ T cell response.

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(9) APPENDICES:

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A cancer vaccine based on wild-type p53

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¹Abbreviations used in this paper: Vp53-wt, vaccinia virus recombinant expressing the full-length murine wild-type p53 protein; HPV, human papilloma virus; RT-PCR, reverse transcriptase-polymerase chain reaction; VRG, vaccinia rabies virus glycoprotein; pfu, plaque forming units.

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ABSTRACT. A vaccinia virus recombinant expressing the full-length murine wild-type p53 protein (Vp53-wt) was constructed and tested in mice for its ability to induce an immune response protective against the growth of tumor cells over-expressing mouse wild-type p53. Mice of 2 different haplotypes immunized with a single dose of the Vp53-wt vaccine were either completely protected or showed delayed onset of tumor formation upon challenge with various types of tumor cells expressing p53. Both CD4⁺ and CD8⁺ T cells, shown to infiltrate tumors of Vp53-wt vaccinated mice, were required for protection. These data suggest a potential use of vaccines expressing wild-type p53 for the active immunotherapy of cancer.

Vaccination is the most effective medical intervention to reduce human morbidity and mortality. While vaccines to infectious agents have been used successfully for many decades, efficacious vaccines for treatment of cancer have been scarce. With advances in molecular genetics, novel targets for cancer vaccines have been identified in recent years. A particularly attractive target for vaccine development might be the p53 protein which is frequently altered in cancer cells. Wild-type p53 acts as a tumor suppressor by curtailing cell cycle progression and inducing apoptotic cell death under certain circumstances (1-2). Mutations which cluster in well-defined regions of the *p53* gene cause structural changes in the p53 protein leading to functional inactivation (3). Most *p53* mutations have been shown to significantly prolong the half-life of the p53 protein and, thus, lead to its overexpression (4). In addition to mutation tumors commonly overexpress wild-type *p53* protein.

A self protein present in excessive amounts in transformed cells, such as mutated or wild-type p53, can trigger a T cell-mediated immune response by exposing so-called cryptic epitopes (5,6) which, when present at physiological levels, have too low affinity to MHC determinants to reach the threshold needed for T cell tolerization or activation.

We tested in a syngeneic mouse model whether mouse full-length wild-type p53 presented by a recombinant vaccinia virus (termed Vp53-wt) induces protection in mice against the growth of spontaneously arisen, transplantable tumor cell lines expressing high levels of endogenous, wild-type p53. Our data show that overexpression of this tumor suppressor protein by a vaccinia virus recombinant elicits a T cell-mediated immune response leading to the complete protection or delayed progression of tumors originating from cells overexpressing p53.

MATERIALS AND METHODS

Mice. Female C57Bl/6 and Balb/c mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained at the animal facility of The Wistar Institute.

Cells. GL261, a murine glioma cell line of C57Bl/6 origin and CT-26. A murine colon adenocarcinoma cell line of Balb/c background were provided by The National Cancer Institute (Frederick, MD). C57Bl/6 fibroblasts were established from C57Bl/6 embryos at 16-18 days of gestation. The t(10)1ras.E7E6 tumor cell line was generated by stable transfection of (10)1 cells, a BALB/c fibroblast line with a spontaneous loss mutation of both p53 alleles (7), with a triple promoter vector (8) expressing v-Harvey-ras under the control of the LTR, the E7 protein of human papilloma virus (HPV)-16 under the herpes simplex virus tk promoter and the E6 protein of HPV-16 under the SV40 promoter. The expression of *p53* transcripts by tumor cells was determined by reverse transcriptase-polymerase chain reaction (RT-PCR). PCR products were sequenced by the Nucleic Acid Facility at The Wistar Institute using a dideoxynucleotide termination reaction. Cells were grown in vitro in DMEM supplemented with 10% FBS and antibiotics.

Vaccinia virus recombinants. The Vp53-wt recombinant was constructed as follows: the DNA fragment encoding mouse full length wild-type *p53* (*p53*-wt) was excised from the pGEM-*p53* vector (9) and cloned into the pSC11 transfer vector for vaccinia virus (10). A recombinant vaccinia virus containing the *p53*-wt sequence was generated by homologous recombination with vaccinia virus strain Copenhagen using thymidine kinase negative cells (11). The recombinant viruses were characterized for the presence of *p53* sequence using PCR. Expression of the *p53* protein was determined by Western blot analysis (12) of *p53* negative (10)1 fibroblasts infected with the vaccinia virus recombinant using Ab-1 and Ab-3 mAbs specific for mouse wild-type *p53* protein (Oncogene, Cambridge, MA). The vaccinia rabies virus glycoprotein (VRG) recombinant,

which is also based on the Copenhagen strain of vaccinia virus, has been described in detail earlier (13).

Immunization and challenge of mice. Tumor cell lines were titrated in syngeneic mice by s.c. inoculation. The tumorigenic doses, defined as the minimal number of cells of either line that causes solid tumors in 100% of animals within ~10 to 40 days, were as follows: GL261: 1×10^5 ; 66.1: 3.7×10^4 ; 410: 4×10^4 and CT-26: 5×10^4 . Protection studies were carried out by injecting 6 to 8 week-old mice s.c. with a single dose of 2×10^7 plaque forming units (pfu) of Vp53-wt in 200 μ l of saline. Control mice were immunized with the same dose of the VRG virus. Mice were challenged 2 weeks later s.c. with tumor cells. Mice were scored as tumor-bearing as soon as tumors were clearly visible. Mice were euthanized once they developed tumors >1.5 cm. Each experiment was repeated 2 to 3 times with comparable results.

Antibody depletion studies. In vivo depletion of specific T-cell subsets was accomplished by injecting mice with the rat mAbs specific for mouse CD4 (i.e., GK1.5, ATCC) or CD8 (i.e., 53.6.7, ATCC), intraperitoneally, using 0.2 ml aliquots of a 1:10 dilution of mouse ascites per mouse. The antibodies were injected on days -4, -1, +1, +4 and +8 relative to tumor cell injection (day 0).

Indirect immunofluorescence. Expression of p53 was determined by immunostaining of cell monolayers with the p53-specific mAb-1 followed with a FITC-labeled goat anti-mouse Ig. The nuclear staining of DNA was performed with propidium iodine. Cells were analyzed by confocal microscopy at The Morphology Core Facility of The Wistar Institute.

Immunohistochemistry. Staining of frozen sections was performed using the avidin-biotin-peroxidase Vectastain Elite ABC kit (Vector Laboratories). Sections were developed with 3',3'-diaminobenzidine tetrahydrochloride (Sigma) and counterstained with 1% hematoxylin. Primary mAbs to CD4 and CD8 (PharMingen) were used to determine infiltration by T cell subsets.

RESULTS

Expression of p53 by the tumor cells. Tumor cells of the different lines were tested for expression of *p53* transcripts and p53 protein. The nucleotide sequence of RT-PCR products from the GL261, 66.1, 410.1 and CT26 cell lines was determined and found to be of wild-type (data not shown). The t(10)1 fibroblast cell line failed to show amplification of a p53 transcript. Indirect immunofluorescence analysis demonstrated varied levels of nuclear p53 expression in GL261 glioma cells, 410.4 mammary adenocarcinoma cells and CT-26 colon adenocarcinoma cells, which overall were substantially higher than those expressed by primary fibroblasts derived from C57Bl/6 embryos. The 66-1 cell line demonstrated uniform staining of 90% of cells (Fig. 1). The t(10)1 cells failed to shown staining with the antibody specific to p53. The differences in levels of expression between normal cells and tumor cells were confirmed by Western blot analysis (data not shown).

The Vp53-wt vaccine induces protective immunity to tumor challenge. To test the efficacy of the Vp53-wt vaccine against growth of GL261 cells, groups of 8-10 C57Bl/6 mice were inoculated s.c. with 2×10^7 pfu of Vp53-wt virus or with the VRG recombinant to ensure that the anti-tumor effect of the Vp53-wt vaccine was caused by a p53 specific immune response rather than by a non specific bystander effect due to an immune response to vaccinia virus. Mice were challenged 2 weeks later s.c. with GL261 murine glioma cells. All of the control mice developed tumors. In contrast, 40-60 % of vaccinated mice showed in several experiments no evidence of tumor growth over a lengthy observation period (Fig. 2). In addition, in animals that developed tumors, mean tumor-free survival time in vaccinated mice was 51.2 days as compared to 28.5 days in control mice ($n=57$, $P<0.00001$). Vp53-wt vaccinated mice that failed to develop tumors upon the initial challenge with 1×10^5 tumor cells were completely protected against a subsequent challenge given 12-15 weeks later with a 5 to 25 times higher dose of tumor cells that caused progressive tumors in 100% of naive mice (Fig. 2). These data indicate that the Vp53-wt vaccine alone induces partial protection against a moderate dose of tumor cells, whereas a

combination of vaccine and low-dose tumor challenge potentiates the immune response thus providing complete protection against an otherwise overwhelming tumor burden.

The Vp53-wt vaccine not only induced a partially protective immune response in C57Bl/6 mice against the GL261 tumor cell line but also in mice of the Balb/c haplotype against murine adenocarcinomas (Table 1). Although complete protection could only be achieved against ~ 30% of mice challenged with the 66.1 mammary carcinoma cells and ~10% of mice challenged with 410.4 mammary and CT-26 colon carcinoma cell lines, all of the Vp53-wt vaccinated groups showed a statistically significant delay in onset of tumors upon inoculation with either tumor cell lines. The protective immune response was specific for p53, since there were not survivors among the vaccinated mice challenged with transformed fibroblast cell line with a loss mutation of both p53 alleles, termed t(10)1rasE7E6, and the increase in median survival did not reach statistical significance (Table 1). On the other hand, growth of this tumor cell line could be inhibited by pre-vaccination with a vaccinia recombinant viruses expressing the E6 or E7 proteins of HPV-16, a viral oncoproteins that are expressed by this cell line (not shown).

The role of CD4⁺ and CD8⁺ T cells in protecting Vp53-wt vaccinated C57Bl/6 mice to challenge with GL261 tumor cells. GL261 tumors excised from mice vaccinated with Vp53-wt or VRG virus were analysed for cells expressing the CD4 and CD8 T cell markers. Tumors of mice vaccinated with the control construct showed a moderate infiltrate with CD4⁺ or CD8⁺ T cells. In contrast, tumors of Vp53-wt vaccinated mice demonstrated a pronounced infiltrate of both T cell subsets throughout the entire tissue sections (Fig. 3). A crucial role for both of these T cell subsets in providing protection against the growth of the GL261 tumor cell line was demonstrated in vivo. Mice vaccinated with Vp53-wt or VRG virus were treated with a regimen of mAbs specific to CD4 or CD8 prior to challenge with GL261 cells given s.c. As shown in Fig. 4A, treatment with either of the antibodies completely abrogated protection and, in addition, accelerated tumor growth. Measuring tumor size over time, mice deficient in CD4⁺ T

cells showed more rapidly growing tumors as compared to mice depleted of CD8⁺ T cells (Fig. 4B).

DISCUSSION

Data presented here show that vaccination with a vaccinia virus recombinant expressing mouse wild-type p53 elicits a specific T cell-mediated immune response that protects mice against a subsequent challenge with tumor cells overexpressing the wild-type form of p53. Vaccination prevents or delays growth of p53 expressing tumor cells in both C57Bl/6 and Balb/c mice. The level of p53 expression did not correlate with the degree of protection; the best protection in terms of complete resistance to tumor growth was achieved in C57Bl/6 mice against the GL261 cell line which showed varied levels of p53 expression (Fig. 1). Protection was less pronounced in Balb/c mice challenged with adenocarcinoma cells which showed pattern and levels of p53 expression similar to GL261 cells (410.4) or uniformly expressed p53 on most cells (66.1). Other parameters are likely to affect the efficacy of the Vp53-wt vaccine. Balb/c mouse strain differs from others (C57Bl/6 and B10.D2), not only in the MHC haplotype and overall genetic background but also in ability to mount efficacious Th1 type immune responses to various pathogens (14) which results from defects in IL-12 receptor signaling in Th2 cells (15). Balb/c mice are more prone to develop Th2 responses, which might influence their ability to eliminate tumor cells upon vaccination to a self protein.

p53 is a tumor suppressor protein that is present in all nucleated cells throughout the life span of an individual. Normal mice should therefore either delete p53-specific T cells in the thymus or develop peripheral tolerance to the dominant epitopes of p53 (16). The p53-specific T cell response observed upon vaccination of C57Bl/6 and Balb/c mice presumably reflects the recognition of cryptic epitopes of p53 revealed to the immune system upon overexpression of the protein by the vaccine.

Vp53-wt-induced protection against tumor challenge required both CD4⁺ and CD8⁺ T cells. The role of CD8⁺ T cells in limiting tumor growth, e.g., by causing direct perforin-mediated lysis of MHC class I⁺ cancer cells, is well defined (17-20). The contribution of CD4⁺ T cells in

eliminating cancerous cells is not as well understood (21-23). CD4⁺ T cells might play a role by secreting cytokines such as IFN- γ , which upregulates MHC class I expression on many tumor cells thus facilitating T cell mediated lysis (24). Other cytokines secreted by activated T cells can initiate inflammatory reactions (25) which can cause tumor cell death. The GL261 cell line does not express MHC class II determinants (data not shown), nevertheless GL261 tumors in Vp53-wt vaccinated mice showed a pronounced infiltration of CD4⁺ T cells which might reflect recruitment of inflammatory MHC class II⁺ cells such as dendritic cells, which upon reprocessing of antigen released by dying tumor cells might cause further activation of T cells. This would explain the increased resistance to high-dose tumor challenge in mice vaccinated to p53 and then challenged with a moderate dose of tumor cells.

Induction of protective immunity by vaccination with wild-type p53 protein had been demonstrated previously. A recombinant canary pox vector expressing human and murine wild-type p53 was previously shown to provide protection in mice challenged with a tumor cell line transfected with a plasmid expressing human p53 (26). Transfected cells express exceedingly high levels of the transgene and, furthermore, human and mouse p53 show extensive sequence heterology within N-terminal region of the protein (27), thus limiting the relevance of this study for cancer therapy. Other studies have shown that synthetic peptides derived from wild-type and mutated murine p53 induce an immune response that protects mice against challenge with a tumor cell line carrying the homologous mutation (28-30). In another study, immunization of HLA-A2.1 transgenic mice with peptides derived from human wild-type p53 was shown to elicit a cytolytic T cell response to HLA compatible human tumor cells. The T cell epitopes were found to be located outside the mutational hot spots (18). These studies together with data presented here clearly show that mutant as well as wild-type p53 can serve as a target antigen for the active immunotherapy of cancer. Although peptides delineated either from mutated or wild-type sequences of p53 might provide a sufficient stimulus to induce an anti-tumor immune response, their usefulness in an outbred human population is limited by the genetic control of peptide:MHC interactions, their high

susceptibility to peptidase-mediated degradation (31), their low immunogenicity, and their inability to induce cytolytic T cell responses (32) unless applied with special adjuvants. Using the entire p53 protein with its numerous potential T cell epitopes should considerably broaden the applicability of a p53 vaccine.

Horror autotoxicus, i.e., induction of auto-immunity, is of obvious concern when using a self protein as an immunogen. Although further studies are needed to test mice vaccinated with wild-type mouse p53 for adverse effects, our experiments to date did not reveal any overt signs of autoimmunity following p53 vaccination indicating that the cryptic p53 epitopes shared between the vaccine and the tumors were not expressed in quantities required for extensive T cell recognition of p53 expressed at physiological levels by normal cells.

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(Figure Legends)

Fig. 1. The nuclear expression of p53 in murine tumor cells immunostained with p53 specific mAb. Staining was assessed under a confocal microscope. Left panel shows immunofluorescence staining of p53, right panel show double staining for p53 and DNA. GL261 glioma (A, B), 66.1 mammary adenocarcinoma (C, D) and embryonal C57Bl/6 fibroblasts (E, F).

Fig. 2. The Vp53-wt vaccine induces partial protection against challenge with GL261 tumor cells. The graph shows results from a representative experiment where groups of 10 C57Bl/6 mice were immunized s.c. with 2×10^7 pfu of Vp53-wt (■) or of VRG (□) recombinant virus and 14 days later challenged with 1×10^5 GL261 cells injected s.c. Mice were observed for over 100 days. Vp53-wt-immune mice that failed to develop tumors after the initial challenge were rechallenged (arrow) with 1×10^6 or 5×10^6 GL261 cells. Mice were observed for an additional 100 days.

Fig. 3. Lymphocytic infiltrates of tumors from mice vaccinated with Vp53-wt (A, C) or VRG (B, D) recombinant virus. Tumors from mice inoculated s.c. with GL261 glioma cells were excised once they reached a size of ~ 1 cm in diameter. Thin sections were analyzed for cells expressing CD4 (A, B) or CD8 (C, D). The number of T cells per 5 mm^2 of tissue section is as follows: A, 500; B, 99; C, 306; and D, 91. (x20).

Fig. 4. Depletion of CD4⁺ or CD8⁺ T cells abrogates the efficacy of the Vp53-wt vaccine. [A] Three groups of 10 C57Bl/6 mice were inoculated with 2×10^7 pfu of Vp53-wt (■) and one additional group was vaccinated with VRG virus (□). Fourteen days later two groups of mice vaccinated with Vp53-wt were treated with mAbs specific to CD4 and to CD8. Mice were challenged with 1×10^5 GL261 cells and the incidence of tumor growth was recorded. [B] The kinetics of tumor growth is expressed as increase in tumor size over time for individual mice treated with mAbs specific to CD4 (▣) or CD8 (▤). The results presented in this graph are from the same experiment as that shown in Fig. 4A.

Table 1. Vaccination with Vp53-wt prolongs tumor-free survival of breast and colon carcinoma challenged mice and does not affect onset of p53 negative tumors.

Cell line	Vaccine	Latency* (days)	complete protection (%)
66.1	Vp53-wt	45.25 \pm 9.1	30
	VRG	28.00 \pm 5.7	
	P* <0.017		
410.4	Vp53-wt	24.57 \pm 1.5	12.5
	VRG	18.85 \pm 3.18	
	P <0.008		
CT-26	Vp53-wt	49.55 \pm 5.85	10
	VRG	39.77 \pm 1.71	
	P <0.003		
t(10)1ras.E7E6	Vp53-wt	16.37 \pm 1.68	0
	VRG	19.22 \pm 2.94	
	P <0.038		

*Values are means s.d.

** Student's t test was used to calculate significance

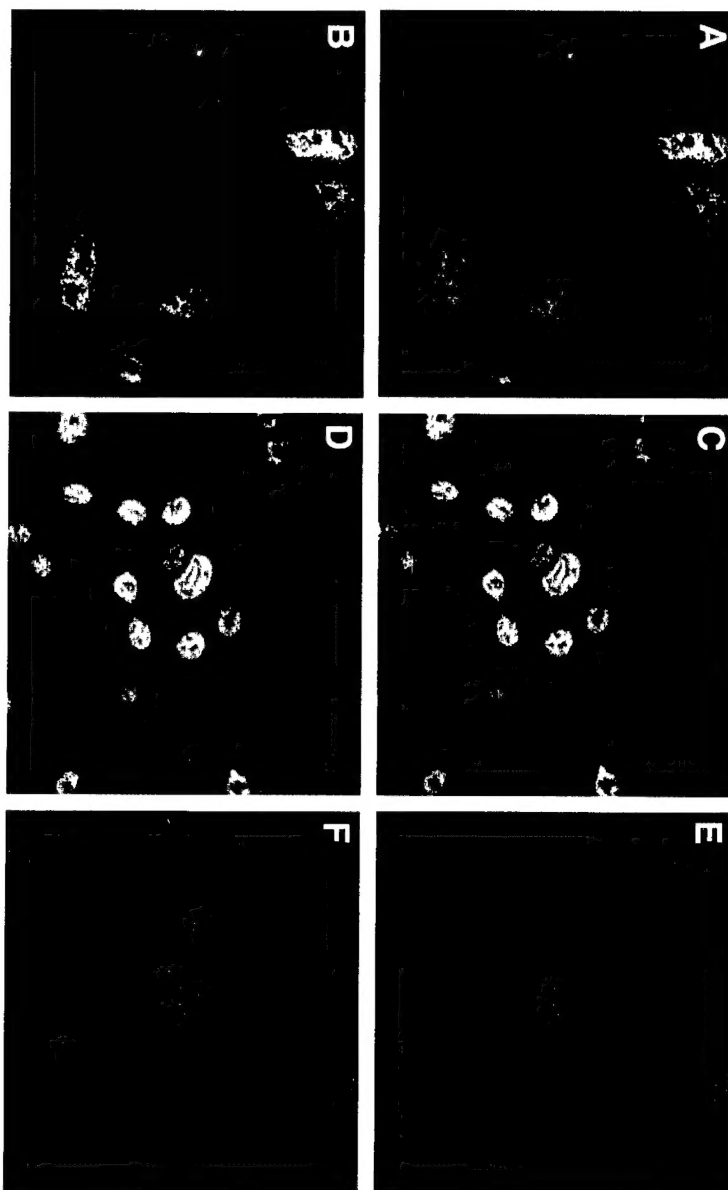


Figure 1

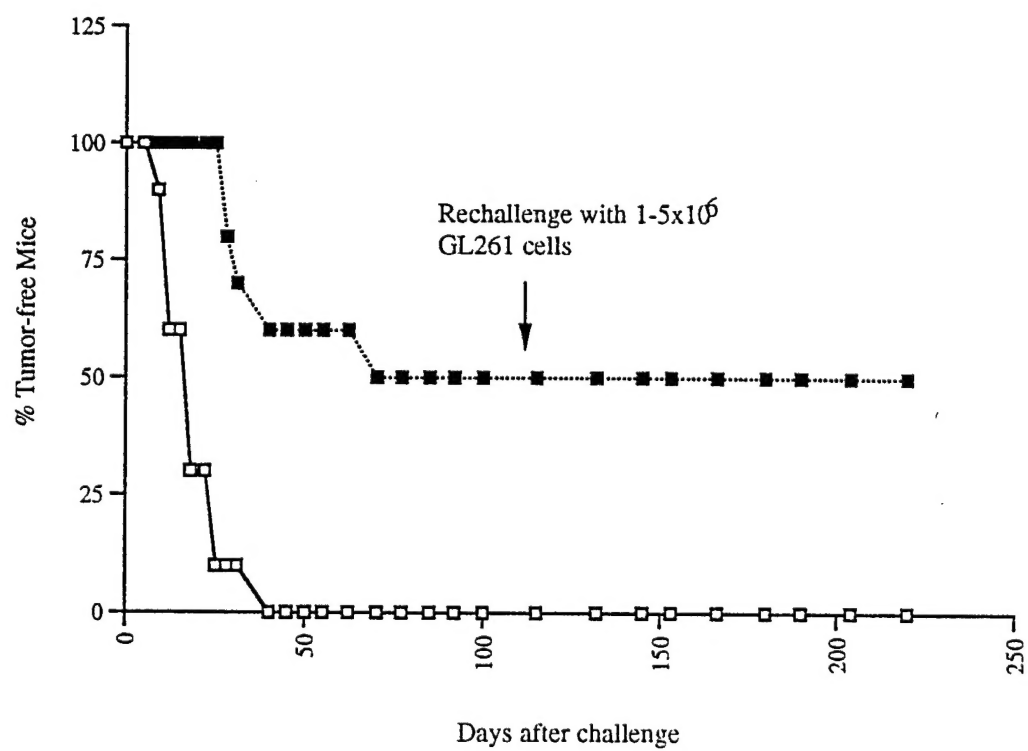


Figure 2

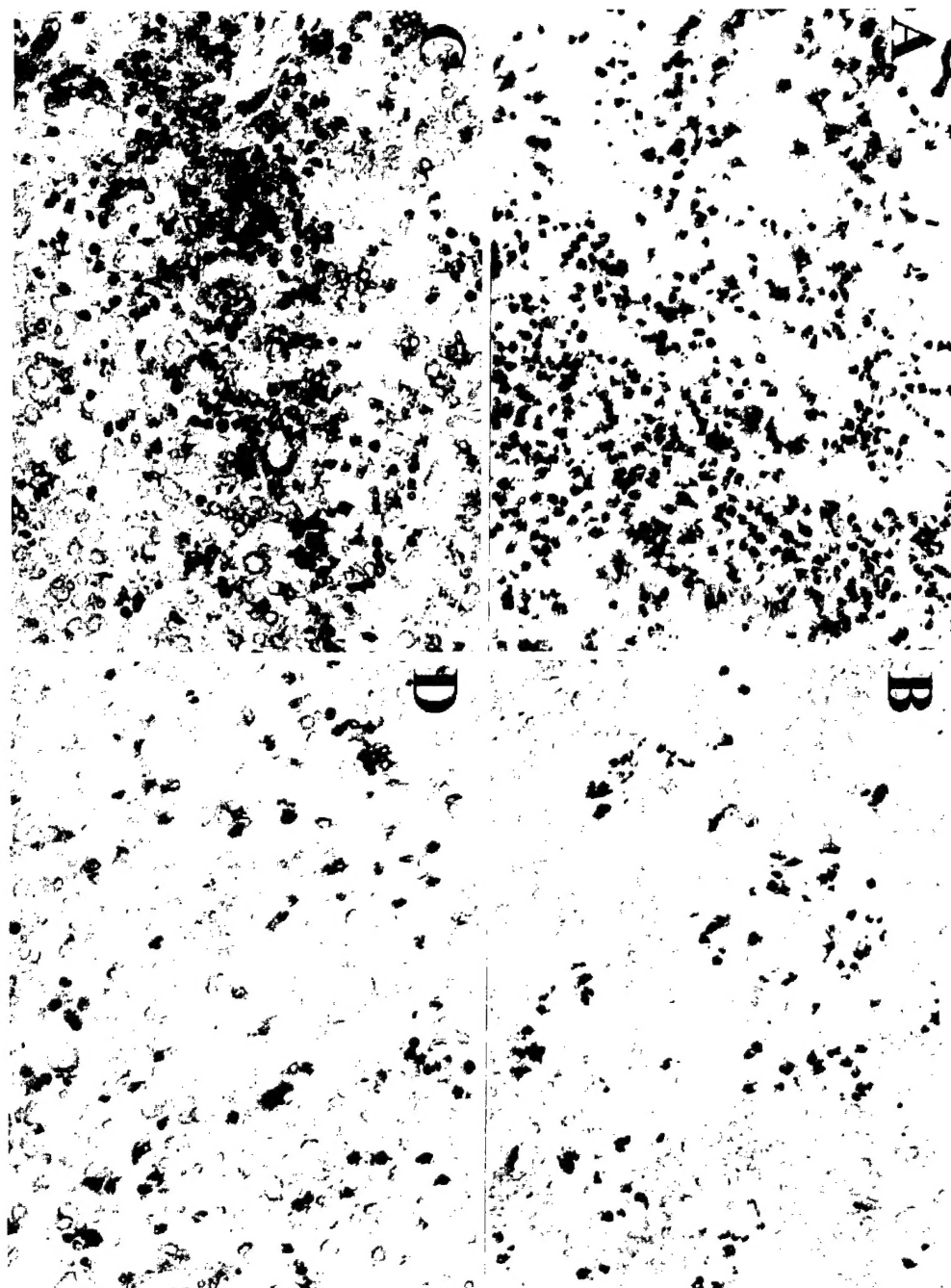


Figure 3

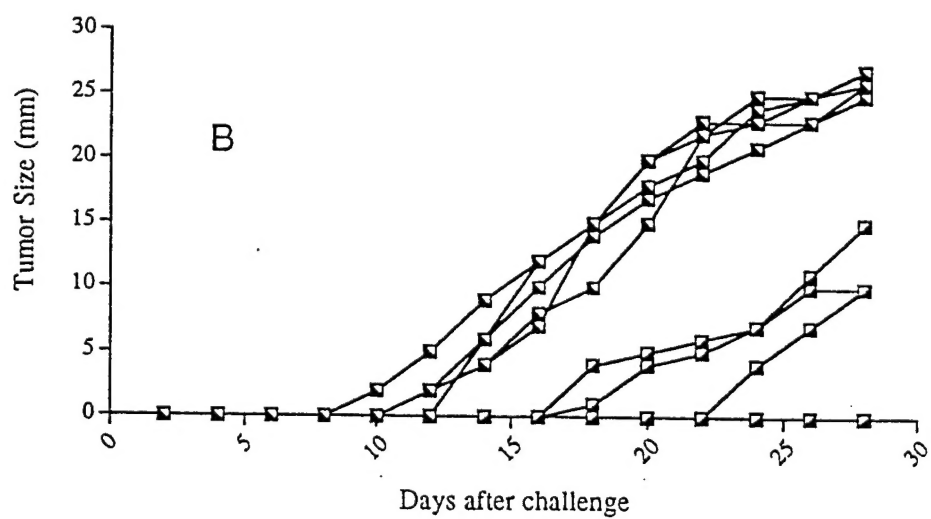
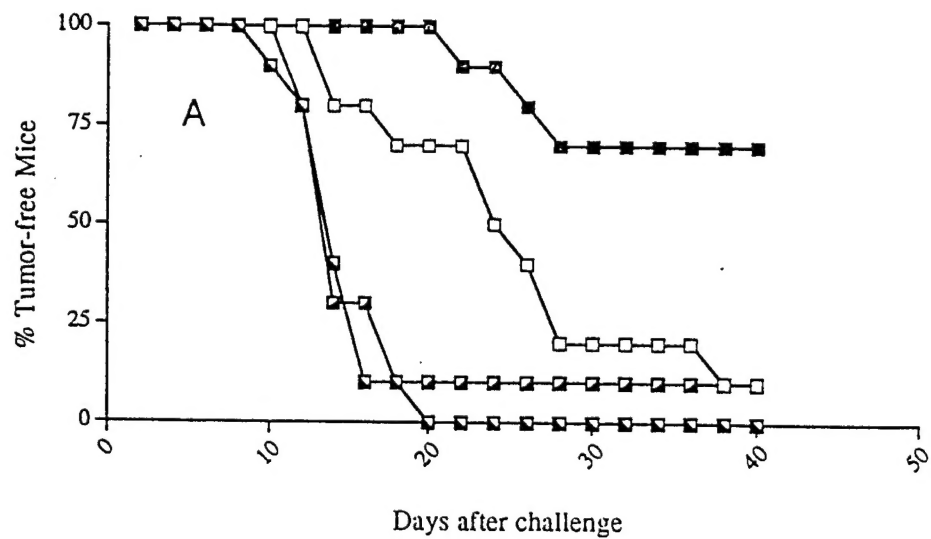


Figure 4